IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In Re Application of
TOIVOLA et al Serial No.
497,813 filed 25th May,
1983 for
"NOVEL TRI-PHENYL ALKANE AND ALKENE DERIVATIVES AND THEIR PREPARATION AND USE"

D E C L A R A T I O N

I, LAURI VEIKKO MATTI KANGAS, declare:

1. That I am a citizen of Finland of Riihipellontie 16 as. 16, 20300 Turku 30, Finland. I am a master of sciences at the University of Turku, and since 1st april 1977 I have been employed by Farmos Group Ltd., the assignees of the above identified application, in research into new drugs. I am co-inventor with Reijo Juhani Toivola, Antero Johannes Karjalainen, Kauko Oiva Antero Kurkela, Marja-Liisa Soderwall, Guillermo Luis Blanco and Hannu Kalervo Sundquist, of the subject matter of the above identified application.

2. The following experiments have been carried out under my supervision to compare the anti-tumour effects of the known anti-estrogenic called tamoxifen with those of 4-chloro-1,2-diphenyl-1-[4-[2-(N,N-dimethylamino)ethoxy]phenyl] 1-butene, hereinafter identified as compound 7 and called compound 7 on page 28 et seq of the above identified application.

Compound 7 is claimed in claim 9 of the said application. Tamoxifen and compound 7 were compared in their ability to surpress DMBA-induced breast cancer in rats and in their ability to surpress mouse uterus sarcoma. The experiments were carried out as follows and the indicated results were obtained.

3. Breast cancer was induced into 45-50 days old female Sprague-Dawley rats by administering a single dose of 12 mg/kg of DMBA (dimethylbenzanthracene) in sesame oil. When using DMBA for the induction the tumours appeared first in the armpits, then on the neck, and furthermore in the sides of the animal. The induction took place in a special isolator (Metall und Plastic GmbH) from which the animals were transfered after about 3 weeks into the animal cages. The treatment began after the appearance of measurable tumours, approximately 5-7 weeks after the induction. The effect of compound 7 on the number of the tumours was studied and compared with that of tamoxifen. The compounds were dissolved in an aqueous solvent containing

polyethylene glycol 3000	28.8 g/l
Tween 80	1.92 "
NaCl	8.65 "
Methyl-p-hydroxy benzoate	1.73 "
Propyl-p-hydroxy benzoate	0.19 "

Compound 7 was administered daily per os to the animals while the controls received the solvent. The treatment lasted for about 40 days. The tumours were detected by individual measurement. The width (w) and the length (l) of the tumours were measured and the volume of the tumour was calculated by the formula: $V = w^2 l/12$. In addition to the size of the tumour, the weight of the animal and the number of tumours were also observed during this study. At the end of the study, when the animals were killed, samples were taken from some animals for histological and hormonal studies. The treatment doses of compound 7 were 0.3, 1, 3, 7.5, 15, 30 and 50 mg/kg. Tamoxifen was used for comparison at doses of 1, 3, and 7.5 mg/kg. Both substances were given per os. The usual number of rats was 4 rats/group. However, the results of various test series have been combined in this study and the group sizes varied greatly.

4. A very good method for evaluating the antitumour effect in the DMBA model cannot be found in the literature. The following criteria were used in this study:

1. The size of the tumour was evaluated by measurement, by comparing the length and the width with a measuring scale in front of the person carrying out the procedure.

2. The volume of the tumour was calculated by assuming the shape of the tumour to be half-oval and by using the formula

$V = w^2 l/12$. When length and width were of the same magnitude, the tumour was assumed to have the shape of a hemisphere. The same formula applies in these cases.

3. The number of tumours was checked at each measurement. Each measurable tumour regardless of size was considered a tumour. If two tumours have grown together, the number of tumours was counted as if the tumours were still separate.

4. When evaluating the size of the tumours, the smallest tumour taken into account had the diameter of 0.3 cm (3 mm). If this size was measured only once, it was not considered a significant growth, because accuracy of measurement in the 0.2 - 0.3 cm range is not good and the effect of errors in the measurement is great on the size of the tumours.

5. The size changes of tumours during treatment was calculated and the tumour changes were classified as follows:

Class 1 = actively growing tumour with at least a four-fold increase in volume.

Class 2 = $\pm$ tumour, the size of which did not change during treatment or the size of which at the end of the treatment was <4-fold compared with the situation at the beginning, or the size of which had decreased during treatment but which even in the end held > 1/4 of its original volume.

Class 3 = regressing tumour, which had decreased to less then 1/4 of its original volume or had disappeared completely.

The numbers of tumours placed in the different categories can be statistically compared by the x^2-test.

6. The numbers of tumours that have totally disappeared were recorded separately.

The t-test and the x^2-test were used in the statistical analysis of the results.

5. The experiments, which are summarized in Tables 1 and 2 below, showed that the number of tumours increased less in the animals treated with compound 7 or tamoxifen than in the control animals. The effect of compound 7 is somewhat more potent than that of tamoxifen. The difference between these two compounds is not, however, statistically significant. The number of tumours disappearing during treatment was greater in the compound 7 group than in the control group and the tamoxifen group. The difference between compound 7 and tamoxifen is significant over a five weeks' follow-up period ($p<0.01$). The results calculated on the basis of tumour growth classification show that compound 7 possesses statistically a very significant antitumour effect compared to the control.

Table 1

The antitumour effect of tamoxifen and compound 7 on DMBA-induced mammary carcinoma in the rat. The number of tumours that appeared and disappeared during five weeks' treatment.

Group	No. of animals	Number of tumours: Start	end	disappeared	change/animal (mean ± sd)	P*
Control	22	83	153	4	3.18 ± 2.95	
Compound 7						
0.3 mg/kg	3	8	12	4	1.33 ± 0.58	NS
1 mg/kg	3	5	6	2	0.33 ± 0.58	NS
3 mg/kg	9	36	42	5	0.67 ± 1.12	<0.05
7.5 mg/kg	8	28	41	1	1.63 ± 2.00	NS
15 mg/kg	5	16	25	4	1.80 ± 2.39	NS
30 mg/kg	4	11	14	3	0.75 ± 1.71	NS
50 mg/kg	1	7	7	0	0.00 ± 0	-
Compound 7 combined results						
0.3-7.5 mg/kg	23	77	101	12	1.04 ± 1.40	<0.01
15-50 mg/kg	10	34	46	7	1.20 ± 1.99	NS
Total	33	111	147	19	1.09 ± 1.47	<0.01
Tamoxifen						
1 mg/kg	7	11	25	2	2.00 ± 0.58	NS
3 mg/kg	15	52	83	0	2.07 ± 1.44	NS
7.5 mg/kg	3	8	9	0	0.33 ± 0.58	NS
Tamoxifen combined results						
1-7.5 mg/kg	25	71	117	2	1.84 ± 1.28	<0.05

* difference from the control group calculated by the t-test.

There is no significant diffrence between tamoxifen and compound 7 at any dose level in respect of the change in the number of tumours/animal.

The number of disappeared tumours is significantly greater in the compound 7 group (19/111) than in the tamoxifen group (2/71) ($p<0.01$, x^2 test).

Table 2

The number of tumours in various groups classified by the growth characteristics. Treatment time 5 weeks. Class 1 = growth > 4-fold, class 2 = size change negligible; class 3 = decrease to ≤ 1/4 of the original or disappearance.

Group	No. of animals	Classification 1	2	3	Comparison to control x^2 (v=2)	P
Control	22	84	65	8		
Compound 7						
0.3 mg/kg	3	6	5	5	14.3	<0.001
1 mg/kg	3	3	1	4	23.1	<0.0005
3 mg/kg	10	17	31	8	11.1	<0.005
7.5 mg/kg	8	16	22	4	3.557	NS
15 mg/kg	5	11	13	5	6.397	<0.005
30 mg/kg	4	5	6	6	19.3	<0.0005
50 mg/kg	1	0	2	5	41.2	<0.0005
Compound 7 combined results						
0.3-7.5 mg/kg	24	42	59	21	16.0	<0.0005
15-50 mg/kg	10	16	21	16	26.4	<0.0005
Total	34	58	80	37	24.1	<0.0005
Tamoxifen						
1 mg/kg	7	9	13	5	7.977	<0.025
3 mg/kg	15	31	47	5	5.740	NS
7.5 mg/kg	3	3	3	3	11.0	<0.005
Tamoxifen combined results						
1-7.5 mg/kg	25	43	63	13	9.404	<0.01

When comparing the diffent dose levels of the same compound, no significant differences were noted, not even when combining the dose levels. The number of strongly diminishing (class 3) tumours is, however, statistically significantly greater in the compound 7 group (37 in class 3, 138 in classes 1 and 2) than in the tamoxifen group (13 in class 3, 106 in classes 1 and 2) ($p<0.025$, x^2 test).

The x^2 values are greater after compound 7 than after tamoxifen. In statistical comparison tamoxifen and compound 7 do not, however, deviate from each other at the dose levels used.

6. Uterus sarcoma was introduced into female NMRI mice by injecting the sarcoma cells in question, suspended in physiological saline, intramuscularly at an amount of 10^6 cells per animal. One hind leg was used as inoculation site. The growth of the tumour was observed by measuring the diameters of the healthy leg as well as of the leg inoculated with the sarcoma cell suspension. The effect of compound 7 on the growth rate of the tumours was studied and compared with that of tamoxifen. The test compounds were dissolved in an aqueous solvent containing

polyethylene glycol 3000	28.8 g/l
Tween 80	1.92 "
NaCl	8.65 "
Methyl-p-hydroxybenzoate	1.73 "
Propyl-p-hydroxybenzoate	0.19 "

Three groups of mice, each group consisting of 6 animals, were used: one for the investigation of tamoxifen, one for compound 7, and one control group. The mice of the control group were given the solvent only. Tamoxifen and compound 7 were both administered perorally at a dose of 1, 10 and 100 mg/kg per day on the following days from the beginning of the test: the 1st, 2nd, 3rd, 9th and the 10th day. Observations on the tumour size were made from the 6th until the 16th day. No antitumour effect with tamoxifen and compound 7 against this tumour was observed at doses of 1 or 10 mg/kg, but at 100 mg/kg positive results were observed, as shown in the following Table.

Table 3

The effects of compound 7 and tamoxifen on the growth of mouse uterus sarcoma.

Individual sizes of the tumours (diameter cm)

Group	Mouse No.	Days 6 after the beginning of the administration	7	8	9	10	13	14	16
Control	1	0.6	0.8	0.8	0.9	1.2	1.9	1.9	1.9
	2	0.7	0.9	1.0	1.1	1.1	1.6	1.6	1.8
	3	0.8	0.9	0.9	1.0	1.2	1.6	1.6	1.8
	4	0.6	0.9	1.0	1.1	1.2	1.6	1.6	1.8
	5	0.6	0.7	1.0	1.1	1.2	1.7	1.7	1.8
	6	0.7	0.8	1.0	1.2	1.6	1.62	1.6	1.9
mean		0.67	0.83	0.95	1.07	1.18	1.67	1.67	1.83
sd		0.08	0.08	0.08	0.10	0.04	0.12	0.12	0.05
Tamoxifen 100mg/kg	1	0.5	0.6	0.9	1.0	1.2	1.7	1.7	1.7
	2	0.6	0.8	1.0	1.1	1.1	1.5	1.5	1.7
	3	0.6	0.8	0.8	0.9	1.1	1.6	1.6	1.7
	4	0.3	0.4	0.6	0.9	1.0	1.1	1.1	1.4
	5	0.6	0.9	0.9	1.1	1.2	1.6	1.7	1.9
	6	0.4	0.6	0.8	1.0	1.1	1.3	1.4	1.7
mean		0.50	0.68	0.83	1.00	1.12	1.47	1.50	1.68
sd		0.13	0.18	0.14	0.09	0.08	0.23	0.23	0.16
difference to control by t-test		<0.05	N.S	N.S	N.S	N.S	N.S	N.S	N.S
~~Compound~~ Compound 7 ~~7~~ 100 mg/kg	1	0.4	0.4	0.7	0.8	0.8	0.8	0.8	0.9
	2	0.6	0.7	0.9	1.1	1.1	1.2	1.4	1.7
	3	0.6	0.7	0.8	1.0	1.2	1.4	1.4	1.5
	4	0.5	0.5	0.6	0.8	1.0	1.2	1.4	1.8
	5	0.3	0.5	0.7	0.9	0.9	0.5	0.5	0.3
	6	0.6	0.7	0.8	1.1	1.1	1.3	1.4	1.7
mean		0.50	0.58	0.75	0.95	1.02	1.07	1.15	1.32
sd		0.13	0.13	0.10	0.14	0.15	0.34	0.40	0.59
difference to control by t-test		<0.05	<0.01	<0.05	N.S	<0.05	<0.01	<0.05	N.S.

7. Mouse uterus sarcoma is an estrogen-receptor-negative tumour, which responds to glucocorticoids. Tamoxifen had no notable effect on the growth rate of the tumours. Compound 7 decreased the growth rate of the tumours in a statistically significant manner at a dose level of 100 mg/kg. This test indicates that compound 7 possesses a cytolytic, non-estrogen-dependent effect.

8. I conclude from these results that compound 7 is at least as effective against the aforesaid hormone-dependent tumours as the known drug tamoxifen. I draw attention particularly to the fact that compound 7 has an effect against mouse uterus sarcoma, a tumour which is known not to be sensitive to estrogens.

31. 10. -84/LK

9. The undersigned declares further that all statements made herein of his own knowledge are true and that all statements made on information and belief are believed to be true; further that these statements are made with the knowledge that wilful false statements and the like so made are punishable by fine or imprisonment or both under Section 1001 of title 18 of the United States Code, and that such wilful false statements may jeopardise the validity of the Application or any Patent issuing thereon.

Lauri Kangas

LAURI VEIKKO MATTI KANGAS

Dated this 31 day of October 1984